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Transforming genes in chronic myelogenous leukemia

(oncogenes/tumor progression/transfection/*RAS*)

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ABSTRACT **Chronic myelogenous leukemia (CML) is a hematopoietic malignancy characterized by an indolent chronic phase that invariably leads to a "blast crisis" indistinguishable from acute leukemia. Using a sensitive assay based on gene transfer and tumorigenesis, we sought evidence that damage to protooncogenes might figure in the progression from the chronic to the blast phase of CML. Seven of the 12 patients with CML examined in this manner harbored transforming genes. Mutations in *RAS* protooncogenes were detected in the leukemic cells from 1 of 6 chronic-phase patients, and 3 of 6 blast-crisis patients. In addition, a presently unidentified transforming gene (neither *RAS* nor *RAF*) was detected in 1 patient with chronic phase and 1 with blast crisis. Our data indicate that mutations in *RAS* genes may play diverse roles in the pathogenesis of CML.**

Chronic myelogenous leukemia (CML) is a hematologic disorder that begins as a protracted chronic phase, characterized by high leukocyte counts and enlarged spleen and liver. In all patients, the chronic phase of CML eventually converts to a "blast crisis" that is indistinguishable from acute leukemia. One molecular change associated with CML is well defined: >90% of patients with the disease have a chromosomal abnormality in their leukemic cells that represents reciprocal translocation between chromosomes 9 and 22 (1). This translocation results in a fusion between two genes known as *BCR* and *ABL* (2, 3). The consistent association of this molecular change with the chronic phase of CML suggests that the fusion between *BCR* and *ABL* is involved in the genesis of CML.

Little is known about what causes the conversion from chronic phase to the blast crisis of CML. Progressive cytogenetic abnormalities involving chromosomes 8, 17, 19, and the t(q9;q22) have been reported, but no molecular descriptions of genetic damage are available. We now report that leukemic cells from some patients with either chronic-phase or blast-crisis CML harbor mutant alleles of *RAS* protooncogenes. The mutations appear to occur more frequently in the blast crisis than in chronic-phase disease. Furthermore, we have detected an additional and presently unidentified transforming gene in single examples of both phases of the disease.

MATERIALS AND METHODS

Cells for Assay. Samples of peripheral blood leukocytes or bone marrow were obtained for extraction of DNA (4).

Tumorigenicity Assay Using Nude Mice. The technique is a modification of that published by Fasano *et al.* (5). Sixty micrograms of high molecular weight DNA was mixed with 4 μg of the plasmid pSV2neo in a sterile Eppendorf tube and ethanol-precipitated from an original vol of 100 μl. The pellet was dried under vacuum, resuspended in 0.9 ml of sterile TE (10 mM Tris·HCl/1 mM EDTA, pH 8.0), and allowed to rock at 37°C overnight. Care was taken to ensure that the DNA was in solution prior to transfection. Samples (0.1 ml) of a 2.5 M $CaCl_2$ stock were added dropwise to the DNA as it was agitated by bubbling through a plugged pipette. This $CaCl_2$/DNA mixture (1 ml total) was added dropwise to 1 ml of 2× Hepes/phosphate buffer (280 mM NaCl/50 mM Hepes/1.5 mM Na_2HPO_4, pH 7.05 at room temperature), again agitating the recipient solution by vigorous bubbling. A flocculent precipitate was allowed to form at room temperature for 20 min. The precipitate (1 ml) was added to a 100-mm dish of 3T3 cells that had been plated with 1×10^6 cells the day before. Eight hours later, the cells were exposed for 90 sec to 15% (vol/vol) glycerol in phosphate-buffered saline. Forty-eight hours later, the cells were trypsinized, replated, and subjected to selection with G418 at 400 μg crude weight per ml of medium. Twenty-one to 30 days after transfection, cells were removed by trypsinization from plates, giving >1000 colonies. Samples of 3×10^6 cells were resuspended in 0.5 ml of cold medium. This suspension was then injected subcutaneously into athymic nude mice, and the emergence of tumors was scored. The observation period for tumor formation was limited to 60 days after injection, since a number of false-positive tumors emerged after this period of time.

Southern Hybridization Analysis. Ten micrograms of high molecular weight DNA was digested overnight with the appropriate restriction enzymes and electrophoresed in 0.8% agarose gels. The DNAs were transferred to Amersham Hybond nylon filters and crosslinked by UV light as suggested by the manufacturers. The filters were prehybridized overnight in 50% formamide/5× SSPE (1× SSPE = 180 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA)/1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.5% $NaDodSO_4$/200 μg of denatured salmon sperm DNA per ml. Hybridizations were performed at 42°C for 24 hr with the same formamide mix as described above, with the addition of radiolabeled DNA probes. After hybridization, the filters were washed in 2× SSC/0.5% $NaDodSO_4$ for 10 min at room temperature, and 1× SSC for 20 min at 68°C twice.

For the detection of codon-12 mutations in *HRAS*, 15 μg of genomic DNA was digested overnight with 50 units of *Msp* I and 50 units of *Hpa* II. The samples were electrophoresed in horizontal 3% NuSieve gels and DNA was transferred onto Zetaprobe nylon filters using 0.4 M NaOH/1.5 M NaCl as transfer buffer. The filters were then neutralized in 2 M Tris·HCl (pH 7.4) for 10 min, washed with 10× SSC at room temperature, and baked at 80°C in vacuum for 2 hr. Prehybridizations were performed in 50% formamide/5× SSPE/1× Denhardt's solution/1% $NaDodSO_4$/200 μg of salmon sperm DNA per ml at 42°C. Hybridizations were done under the same conditions, but with the addition of

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Abbreviation: CML, chronic myelogenous leukemia.

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labeled probe at 5×10^6 cpm/ml. The probe used was a 600-base-pair (bp) *Sma* I fragment from plasmid pEJ (6). The filters were washed with 2× SSC/0.5% $NaDodSO_4$ at room temperature, then twice at 50°C for 15 min each.

Molecular Cloning of a Mutant *NRAS* Allele. DNA from a nude mouse tumor arising from a secondary transfection was digested to completion with *Eco*RI. Fractions enriched for 7- to 9-kilobase (kb) fragments were collected, and genomic libraries were constructed with this pool using the λgtWES vector. Inserts from selected clones positive on secondary screening with a human *NRAS* probe were digested with *Hin*dIII (to isolate the first exon) or *Bst*EII/*Pst* I (to isolate the second exon), subcloned into M13, and sequenced by the dideoxy chain-termination method (7).

Gel Hybridization Assay. Gel hybridizations were performed according to Bos *et al.* (8), using the oligomer AACACCACTTGCTCCAAC as probe, which is specific for the codon-12 mutation in *NRAS*. The washes subsequent to hybridization were all in 6× SSC: 4 times at 4°C, twice at 50°C for 1 min each, and once at 62°C for 1 min.

RESULTS

Transforming Activity of DNA from Patients with Blast-Crisis CML. DNA from peripheral blood leukocytes or bone marrow from four of six patients with CML in blast crisis exhibited transforming activity in the tumorigenicity assay (Table 1). The tumors arising in nude mice became apparent between 14 and 58 days after injection of transfected cells, and in all cases the tumors bore human repetitive (*Alu*) DNA when analyzed by Southern blot hybridization (data not shown). This indicates that tumorigenesis was associated with the transfer of human DNA. A second cycle of transfection with DNA from primary nude mouse tumors again gave tumors that carried human *Alu* sequences. Since random segregation of transfected human DNA with the tumorigenic phenotype through several cycles of transfection is highly unlikely, the data strongly suggested that the transforming principle was carried in human DNA.

Mutations in the *RAS* family of protooncogenes (*HRAS, NRAS, KRAS*) appear frequently in acute leukemias (9, 10). Therefore, we asked whether the transforming genes isolated from blast crisis were of this family. Using gene-specific probes, we determined that *RAS* genes were responsible for the activity of three of the four DNA samples that scored in the tumorigenicity assay (two *HRAS* and one *NRAS*) (Table 1). One sample (T6A-8-22-86, primary nude mouse tumor from transfections using DNA from patient Jac), however, bore a transforming gene that was not in the *RAS* family. The presence of a human transforming gene was confirmed with secondary transfections, which gave tumors that again carried human repetitive DNA. Artifactual rearrangements during transfection of the *RAF* gene may activate it to a transforming allele (11). Southern blot analysis of the nude mouse tumors from Jac, however, showed no hybridization with a *RAF* probe. We have yet to test Jac DNA for other protooncogenes that are occasionally activated by artifacts in transfection assays.

Transforming Activity of DNA from Patients with Chronic-Phase CML. DNAs from hematopoietic cells of patients with chronic-phase CML showed transforming activity in two of six cases (Table 1). Tumors arose in nude mice within 20–40 days, and secondary transfections yielded tumors bearing human *Alu* sequences. In the nude mouse tumor from one case (tumor T3A-28-8-86R from transfections using DNA from patient Re), the transforming allele was identified as *HRAS*; in the other case (tumor T6B-2-8-86 from transfections using DNA from patient Sch-CP), the gene was neither a *RAS* gene nor *RAF*.

Mutations of *RAS* Genes in CML. Activation of *RAS* genes usually results from mutations in codons 12 or 61 (12). We sought to determine the nature of the mutations in the active *RAS* genes detected by transfection with CML DNA. Codon-12 mutations in human *HRAS* can be detected by cleaving DNA with *Msp* I and *Hpa* II. The normal *HRAS* gene contains the sequence GCCGGC encoding for 11th and 12th codons. Both *Msp* I and *Hpa* II recognize the tetramer CCGG and therefore will cut the *HRAS* gene within the sequence encoding the 12th codon. This will generate a

Table 1. Transforming genes in DNA from blood cells of CML patients

DNA source	Philadelphia chromosome	Tumorigenicity of transfected cells*	Latency, days	Transforming gene
T24	0	7/7	14–21	*HRAS*
Normal human DNA	0	1/12	45	—
CML blast crisis†				
Per (M)	+	1/4	40	*NRAS*
Car (M)	+	2/2	14	*HRAS*
Sab (L)	+	2/3	34–58	*HRAS*
Fop (M)	+	0/5	—	—
Jac (M)	+	2/7	40–48	*X*‡
Sch-BC (M)	+	0/4	—	—
CML chronic phase				
Jo	+	1/4	60	—§
Chr	0	0/3	—	—
Re	+	2/2	13–16	*HRAS*
The	+	0/5	—	—
BL19330	+	0/4	—	—
Sch-CP	+	1/3	40	*X*¶

*The observation period for tumor formation was 60 days after injection. First figure specifies number of transfections that produced tumors, second figure specifies total number of transfections tested in tumorigenicity assay.

†(M), myeloid blast crisis; (L), lymphoid blast crisis.

‡*X*, unidentified transforming gene as described in the text. Two of three secondary transfections resulted in tumors with latency periods of 30–38 days.

§No *Alu*-positive tumors developed in seven attempts using the primary nude mouse tumor DNA. This suggests that no human gene was responsible for the tumorigenicity.

¶One of four secondary transfections resulted in tumors with a latency period of 58 days.

355-bp fragment. Mutations within codon 12 obliterate this recognition site and result in a 412-bp fragment. Thus, the normal and a mutant allele in codon 12 can be discerned on Southern blot hybridization analysis. Using this method, we found that all the *HRAS* alleles responsible for nude mouse tumors had mutations in codon 12 (Fig. 1). We extended this analysis to DNAs from hematopoietic cells of the CML patients and identified the presence of codon-12 mutations in these primary samples (Fig. 2).

We sought to identify the mutation in *NRAS* from our patient with CML blast crisis. Since no convenient restriction polymorphisms are available for *NRAS*, the first and second exons of the transforming allele were cloned from a secondary nude mouse tumor and sequenced. The data revealed that the activating mutation was in codon 12, converting the sequence GGT to AGT, and thus substituting serine for glycine in the *NRAS* protein. We then wished to ascertain whether the same *NRAS* mutation existed in the patient's leukemic blood cells. Using hybridization with an oligomer probe specific for the codon-12 mutation, we determined that the codon-12 mutation in *NRAS* (AGT) was present in the patient's leukemic cells (Fig. 3).

An Unidentified Oncogene Isolated from Both the Chronic Phase and Blast Crisis of CML. DNAs from the leukemic cells of patient Sch-CP (CML chronic phase) and patient Jac (CML blast crisis) both gave tumors in nude mice at low efficiencies (Table 1). Secondary transfections with DNA from the primary nude mouse tumors resulted in tumors that carried human *Alu* sequences, and the transforming gene was neither *RAF* nor *RAS*. The patterns of human DNA in the secondary tumors, however, suggested that the transforming genes in Sch-CP and Jac were the same: when the secondary tumors were digested with *Eco*RI, *Hin*cII, *Pst* I, or *Pvu* II, multiple common bands were detected with each enzyme (Fig. 4). From the sum of the sizes of the common bands, we estimated the length of the putative transforming gene to be between 23 and 35 kb. Until further molecular characterization has been completed, we will call this gene *X*.

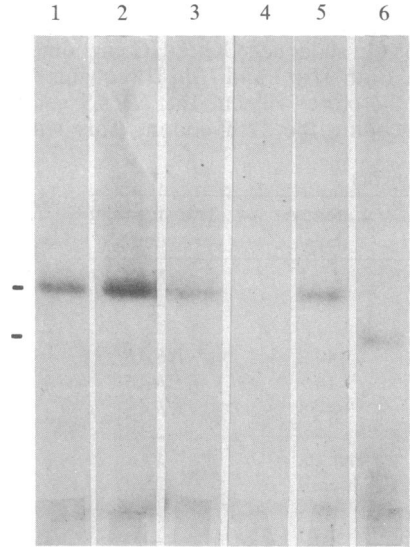

FIG. 1. Mutations in codon 12 of *HRAS*. DNA samples were digested with *Hpa* II/*Msp* I and electrophoresed in a 3% NuSieve gel. The probe was the 0.6-kb *Sma* I fragment from pEJ. The slower-migrating band is 412 bp and represents the *HRAS* allele mutated in codon 12. The faster-migrating band is 355 bp and represents the normal *HRAS* allele. DNA sources: lane 1, 1BEJ, mouse tumor arising after transfection with T24 DNA, which harbors a codon-12 mutation in *HRAS*; lane 2, T7A-10-22-85, nude mouse tumor from transfection with Car DNA, CML blast crisis; lane 3, T7-2-8-86L, nude mouse tumor from transfection with Sab DNA, CML blast crisis; lane 4, a nude mouse tumor with no mutations in *RAS*; lane 5, T3A-7-23-86, nude mouse tumor from transfection with Re DNA, CML chronic phase; lane 6, K562 cell line.

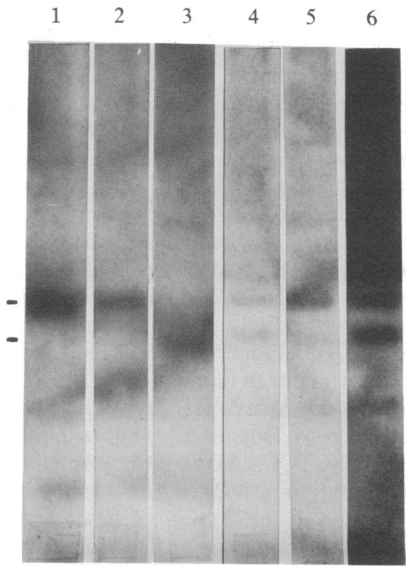

FIG. 2. Codon-12 mutations in *HRAS* of patients with CML. DNA samples were treated as described in Fig. 1. DNA sources: lane 1, 1BEJ, nude mouse tumor arising from transfection with T24 DNA, which harbors the *HRAS* allele mutated in codon 12; lane 2, T24 bladder carcinoma cell line; lane 3, K562 cell line, which bears no mutant *RAS* allele; lane 4, Re, CML chronic phase; lane 5, Car, CML blast crisis; lane 6, Sab, CML blast crisis.

Search for Amplification of Protooncogenes in the DNAs from CML Patients. Southern blots of DNA from patients' blood cells were hybridized with probes for *ABL*, *ERBB2*, *HRAS*, *KRAS*, *NRAS*, *MYC*, *MYB*, and *RAF*. None of these genes was amplified in leukemic cells (data not shown).

DISCUSSION

Mutations of *RAS* Genes in CML. Little is known of the genetic events that accompany and possibly cause transition from the chronic phase to the blast crisis of CML. Here we report the testing of six patients with chronic-phase disease and six with blast crisis for the presence of activated oncogenes in their leukemic cells. Using a sensitive tumorigenicity assay (5), we detected transforming genes in two of the six patients with chronic phase CML and in four of six

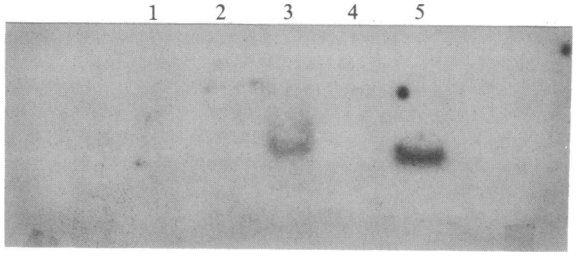

FIG. 3. A codon-12 mutation in *NRAS* of the blood cells from patient Per. Genomic DNAs were cleaved with *Pst* I and electrophoresed in a 0.8% agarose gel. The probe used was a 20-nucleotide oligomer specific for the codon-12 mutation in *NRAS*. DNA sources: lane 1, K562 cell line; lane 2, HL60 cell line; lane 3, Per, CML blast crisis; lane 4, T9-8-28-85, nude mouse tumor bearing a mutant *HRAS* allele; lane 5, T2B-6-30-86, secondary nude mouse tumor arising from transfections with Per DNA. The increased intensity of the hybridizing band in lane 5 is due to amplification of the transforming *NRAS* gene in the nude mouse tumor.

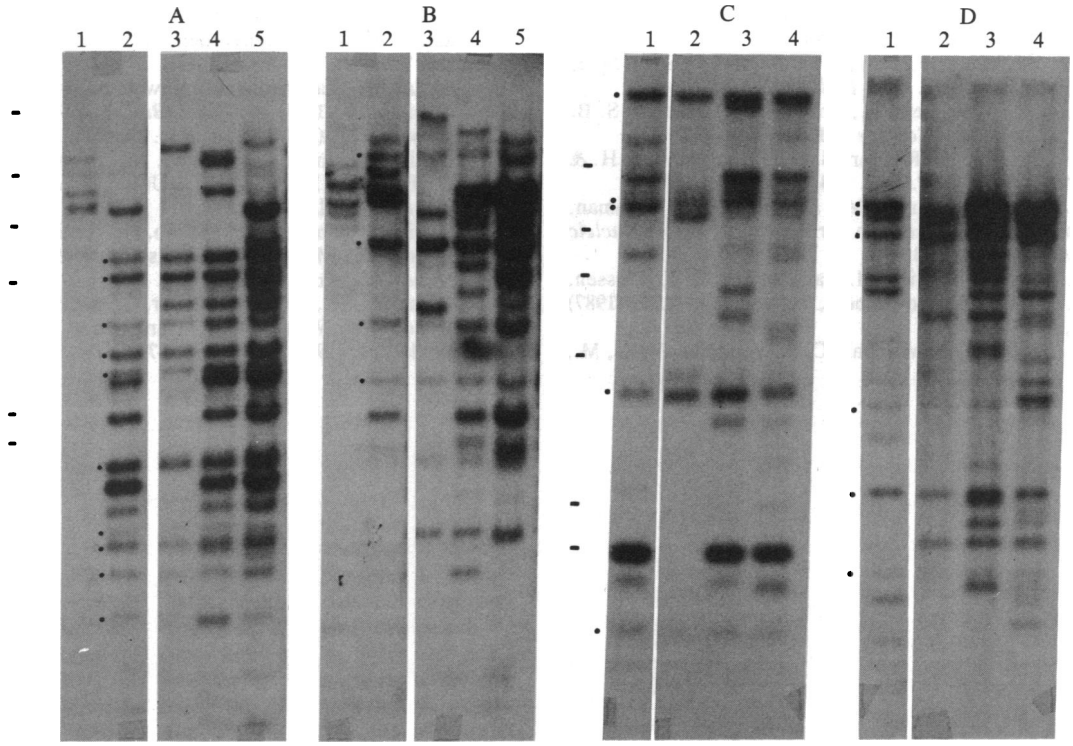

FIG. 4. Comparison of unidentified transforming genes from two individuals with CML. DNAs were cleaved with *Pst* I (*A*), *Pvu* II (*B*), *Hin*cII (*C*), or *Eco*RI (*D*). Hybridization was performed with a probe for human *Alu*. In *A* and *B*, the sources of DNA were as follows: lane 1, T1A-9-22-86, arising from transfections with DNA bearing a mutant *KRAS* allele; lane 2, T4-7-22-86, arising from transfections with Sch-CP DNA; lane 3, T3B-3-9-87d, first nude mouse tumor arising from transfections with Jac DNA; lane 4, T3B-3-9-87a, second nude mouse tumor arising from transfections with Jac DNA; lane 5, T7-3-9-87, third nude mouse tumor arising from transfections with Jac DNA. In *C* and *D*, DNA sources were as follows: lane 1, T4-7-23-86, nude mouse tumor arising from transfections with Sch-CP DNA; lane 2, T3B-3-9-87d, first nude mouse tumor arising from transfections with Jac DNA; lane 3, T3B-3-8-87a, second nude mouse tumor arising from transfections with Jac DNA; lane 4, T7-3-9-87, third nude mouse tumor arising from transfections with Jac DNA. Dashes designate the position of marker fragments from λ phage DNA cleaved with *Hin*dIII. Dots designate DNA fragments bearing *Alu* sequences that are shared by samples derived from patients Sch-CP and Jac.

patients in blast crisis. Four of the six transforming genes were alleles of *RAS* genes (*HRAS* or *NRAS*), with mutations affecting codon 12—one from chronic-phase disease and three from blast crisis. Hirai and colleagues (13) reported a mutant allele of *NRAS* in one of eight patients with chronic-phase CML. In combination, the available data suggest that mutations of *RAS* genes may be more common in blast crisis than in chronic-phase CML, but larger studies will be necessary to test this impression properly.

We hypothesize that mutations in *RAS* genes may on occasion contribute to the transition from chronic-phase CML to the blast crisis. The relative frequency of activated *RAS* genes in samples from blast crisis is in accord with this hypothesis, although the sample size is too small to be decisive. The occasional presence of mutant *RAS* genes in the chronic phase indicates that mutations in *RAS* genes can also be earlier events in leukemogenesis. There is a precedent for this view in recent findings with myelodysplasia, a preleukemia in which mutant alleles of *RAS* genes have been found in hematopoietic cells as early as 1.5 years prior to conversion to acute leukemia (4, 14).

An Additional Transforming Gene in CML. We have detected a presently unidentified transforming gene in single cases of chronic-phase CML and blast crisis. The gene is not a member of either the *RAS* or *RAF* families of protooncogenes, but nothing further is known of its identity. The gene has a relatively low transforming potential, giving tumors in only 3 of 10 attempts, with a latency of 40–48 days (see Table 1). Sequential passages of the activated gene through assays for tumorigenesis did not enhance its activity, in contrast to results with activated alleles of *RAS* genes.

Some protooncogenes can be activated by artifactual rearrangements that occur during the manipulations required for the bioassay used here (11, 15, 16). The artifact has affected *RAF* with special frequency (11) but is a rare event in all instances. The unidentified gene reported here has been detected in three separate transfections, using DNA from two different patients. It therefore seems unlikely that the gene has been activated by an artifact. Further analysis will be required to sustain or refute this deduction.

CML as a Model for Tumor Progression. The natural history of CML makes the disease an attractive setting for the study of human tumor progression. Although work reported here failed to identify decisively any genetic factor that might contribute to progression in CML, our results exemplify two points: first, that tumorigenesis characteristically arises from combinations of genetic lesions; and second, that these lesions can play diverse roles in tumorigenesis—in the present instance, it appears that mutant *RAS* genes may figure in either the pathogenesis of chronic-phase CML or progression to blast crisis.

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